Metabolic Behavior of Pseudomonas savastanoi Isolates from Olive and Oleander on Certain Carbohydrate and Amino Substrates

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Supported by PHS Research Grant AI-06323 from the National Institutes of Health.

Accepted for publication 22 October 1971.

ABSTRACT

Pseudomonas savastanoi from tumors on olive and oleander did not differ significantly in oxidative metabolic patterns on twelve substrates. When cells were cultured in a medium with D-glucose as the carbon source, oxygen absorption in respirometer experiments was the highest on alanine, followed in descending order by fructose, serine, glucose, glycerol, glutamine, asparagine, and galactose. Oxygen uptake was scant or zero on sucrose, xylose, phenylalanine, and tryptophan. On a complete medium containing sucrose, however, most oleander isolates grew and produced measurable amounts of acid. Such isolates grown in a sucrose medium readily oxidized sucrose in respirometer tests. An inducible sucrose transport system and an inducible β-fructofuranosidase (β-D-fructo-furanoside fructohydrolase, E.C. No. 3.2.1.26) allows utilization of sucrose by these isolates. With one exception, olive isolates that were tested apparently lack the capacity to produce both systems.

Phytopathology 62:349-355.

Additional key words: sucrose metabolism, induction of β-fructofuranosidase.

Smith (12), Adams & Pugsley (1), and Pinckard (10) reported that *Pseudomonas savastanoi* and the bacterium which produces aerial tumors on oleander were similar in the morphological, cultural, and physiological features studied. In common with earlier investigators (11, 13), none of these workers was able to infect the oleander with the olive pathogen. Apparently because of such results, the 1957 edition of *Bergey's Manual of Determinative Bacteriology* listed the oleander pathogen as *Pseudomonas tonelliana* (Ferr.) Burkh. Sutic & Dowson (14, 15), who also failed to infect the oleander with the olive pathogen, followed the recommendations of Smith (12) and designated the oleander pathogen "*Pseudomonas savastanoi* var. *nerii*".

In contrast, Wilson & Magie (23) found that certain isolates from olive produced tumors on oleander similar to those produced by oleander isolates. Furthermore, no marked differences between olive and oleander isolates were revealed by additional physiological and serological tests (4, 6, 21, 22, 23).

This study examined the olive and oleander pathogens as to oxidative metabolic rates on various carbohydrate and amino substrates. Meyer & Cameron (7, 8), using such a method with *Brucella* species, found that each species displayed "a characteristic and definitive oxidative metabolic pattern," and that each of several "strains" which were unassignable to a particular species by conventional determinative methods, exhibited the oxidative metabolic pattern of one of the established species.

MATERIALS AND METHODS.—Cultures of the bacteria were obtained from oleander and olive plants in several locations in California. Many were progeny of single cells; the rest were obtained through the single-colony dilution method. Each isolate that was tested for pathogenicity infected the type of host (olive or oleander) from which it was obtained.

For testing oxidative metabolism, the bacteria were grown for 18-20 hr in 300 ml of a liquid medium at 25 C on a shaker. The culture medium (glucose medium A) was composed of 3.0 g casamino acid, 0.3 g magnesium sulfate, and 10 g glucose/liter of 0.01 M potassium phosphate buffer (pH 7.5). In some specified experiments, 10 g of sucrose (sucrose medium A) or a combination of 5 g of D-glucose and 5 g of sucrose (glucose-sucrose medium A) were substituted for 10 g of D-glucose in this medium.

The cells were centrifuged at 8,000 *g* for 5 min at 4 C, and the sedimented cells were then suspended in 0.85% sodium chloride solution that was buffered with 0.05 M potassium phosphate, pH 7.5. The cells were precipitated by centrifugation as before, and resuspended in the buffered sodium chloride. The suspension was adjusted to an absorbance of 0.3 to 0.5 at 620 nm in a Zeiss PQM II spectrophotometer, and a sample was taken for nitrogen determination by the micro-Kjeldahl and nesslerization methods (2).

Respirometer experiments were carried out in a Gilson differential respirometer by procedures listed in Umbreit et al. (17). Each flask contained 1 ml of the bacterial suspension, 1.5 ml of 0.01 M potassium phosphate buffer, pH 7.5, and 0.5 ml of a solution containing 10 mg/ml of the desired substrate. After an equilibration for 30 min at 25 C, oxygen uptake was recorded at 20-min intervals for 1 hr.

The results were expressed as QO_2N, the number of microliters of oxygen absorbed per hr per mg of bacterial nitrogen minus the endogenous rate. All values falling within the range of plus or minus two standard deviations ($\sigma = \sqrt{N}$) were considered to conform to a normal distribution (18).

The medium (medium B) used in the study of sucrose utilization consisted (per liter) of: 1.69 g ammonium sulfate; 0.39 g dibasic potassium phosphate; 5 g sodium chloride; 0.03 g bromothymol blue; and 15 g agar. The basal medium was adjusted to pH 7.1 and sterilized for 45 min in an autoclave, and 15 g/liter of the desired sugar (sterilized by filtration) were added. Fifteen-ml portions of the medium were dispensed in 25- X 200-mm test tubes, and slants prepared. The bacteria, in saline suspension, were spread over the surface of the agar, and the tubes were incubated at 25 C.

Enzyme preparation.—Cultures were centrifuged for 5 min at 8,000 *g* at 4 C. The sedimented cells were suspended at the rate of 1 g wet wt/3 ml of cold 0.05 M potassium phosphate buffer, pH 7.0, and subjected to four 15-sec exposures to sonic oscillation (Bronwill Biosonic III). Samples then were centrifuged at 20,000 *g* for 15 min at 0 C. The supernatant liquid was dialyzed against 2 liters of water at 4 C for 16 hr, then used as the source of enzyme.

For induction experiments, cells grown for 18 hr at 25 C in 1 liter of glucose medium A were centrifuged at 8,000 *g* for 5 min under aseptic conditions. The cells were washed twice with 500 ml of sterile culture medium A with the carbon source omitted, then suspended in 1 liter of culture medium A with glucose or sucrose or both as the carbon source. The suspensions then were shaken at 25 C. At various intervals, 250-ml portions of the cell suspension were removed, processed for enzyme preparation by the procedures described above, then assayed for β-fructofuranosidase.

β-fructofuranosidase assay.—Enzyme preparation (0.25 ml), 0.05 M, pH 7.0 potassium phosphate (0.25 ml), and 0.8 M sucrose (0.1 ml) were incubated at 23 C for 10 min, then heated in boiling water for 5 min to stop enzyme activity. The heated samples were cooled to 23 C, then centrifuged at 5,000 *g* for 10 min at 23 C. The supernatant liquid was assayed for glucose with glucose oxidase (Glucostat Special, Worthington Biochemical Corp.) following the manufacturer's specifications. For specific activity determinations, the concentration of enzyme preparation was adjusted to give a linear reaction rate over the 10-min incubation period. A unit of β-fructofuranosidase activity is the amount of enzyme that catalyzes the release of 1 μmole of glucose/min under the conditions of the assay described above. Specific activity is expressed as units of β-fructofuranosidase activity per mg of protein as determined by the biuret procedure (5).

Detection of the products of enzyme action.—Enzyme preparation, 0.25 ml; 0.05 M, pH 7 potassium phosphate buffer, 0.25 ml; and 0.1 ml of sucrose-U-^{14}C, 0.8 M, specific activity, 12.5 μc/mmole, were incubated at 23 C for varying periods, then heated in boiling water for 5 min to terminate enzyme action. The reaction mixtures were cooled and centrifuged at 5,000 *g* for 5 min. Ten μliters of the supernatant liquid were chromatographed alongside authentic samples of glucose and fructose on Whatman No. 1 filter paper with *n*-butanol-glacial acetic acid-water (4:1:5, v/v, organic phase) as the solvent. Radioactive compounds on the chromatograms were detected by a Nuclear Chicago chromatographic strip counter.

Portions bearing radioactive compounds were cut from the chromatograms and placed in vials of scintillation counting fluid (9). The vials were counted by a scintillation counter, and the amounts of substrate utilized and products formed were calculated from the radioactivity associated with the compounds.

Glucose and fructose were detected on the chromatograms with silver nitrate (16).

Characterization of the products of sucrose degradation.—Supernatant fractions from reaction mixtures described above were freeze-dried, and the residue from each sample was suspended in 0.2 ml of water. The entire sample was applied to Whatman No. 3 MM filter paper, and the chromatogram developed in the solvent described above. The radioactive areas on the chromatogram bearing the products of enzyme action were cut out and eluted with water, and the eluate was freeze-dried. The residue of each sample was dissolved in 1 ml of water. Then 6 mg of anhydrous sodium acetate and 0.1 ml of phenylhydrazine reagent (0.1 g phenylhydrazine/ml 10% acetic acid) (20) were added, and the samples heated in a boiling water bath for 15 min. The treatment converts hexose sugars to their corresponding osazones (20). After the samples had cooled, the crystals that formed were collected by filtration and dried in a desiccator. Although D-glucose and D-fructose form the same osazone (19, 20), the two sugars are separated by paper chromatography prior to reaction with phenylhydrazine.

Products of sucrose degradation were also identified and quantitatively measured by assays

specific for D-glucose and D-fructose that utilize hexokinase, phosphohexoisomerase, and glucose-6-phosphate dehydrogenase (3).

Sucrose transport studies.—Cultures grown 24 hr in 500 ml of medium A with glucose as a carbon source were divided into four 100-ml samples. Each lot was centrifuged at 8,000 *g* for 5 min at 4 C, then suspended in glucose medium A. The cells were centrifuged as before, and the precipitated cells suspended in glucose medium A, glucose-sucrose medium A, or sucrose medium A. These suspensions were incubated with shaking at 25 C. After 90 min, each cell suspension was centrifuged at 8,000 *g* for 5 min at 4 C. The cells of all samples were washed by suspending them in medium A without a carbon source and then centrifuging the suspensions at 8,000 *g* for 5 min at 4 C. The cells were washed once more, then suspended to a final concentration of 2 mg/ml (dry weight) in medium A without a carbon source. Reaction mixtures were prepared by mixing 0.1 ml of the bacterial suspension and 1.4 ml of medium A containing 0.15 μmole of sucrose-^{14}C (uniformly labeled) Schwarz Bioresearch, specific activity, 0.43 μc/μmole. After 30 sec, 1 ml of each reaction mixture was passed through a Millipore filter, pore size, 0.45 μ. The filter pad was immediately rinsed with 5 ml of the sucrose medium A at 25 C. The filter pads were removed, air-dried, and placed in 5 ml of scintillation fluid (9). Radioactivity was measured with a liquid scintillation counter.

RESULTS.—*Oxygen uptake.*—We first determined the oxidative rates of 25 of our stock isolates, about half from olive and half from oleander. The substrates were five carbohydrates, six amino acids, and glycerol. Oxygen uptake was highest for both olive and oleander isolates on alanine, followed in descending order by fructose, serine, glucose, glycerol, glutamine, asparagine, and galactose (Table 1). Values for sucrose, xylose, phenylalanine, and tryptophan were practically negligible. Whether or not sucrose can be utilized by the organism as an energy source is discussed later in the paper. Twenty-five single-colony subisolates of each of a new olive and a new oleander culture were tested on glucose, fructose, alanine, serine, and asparagine. Here again, oxygen uptake was greatest for alanine, followed in descending order by fructose, serine, glucose, and asparagine.

Whether or not there were significant differences between olive and oleander isolates is not clear from these results. Whereas, in the case of the stock cultures, the oleander isolates gave lower values than olive isolates on glucose, the reverse was true for the subisolates. Whereas among the stock cultures the value for oleander isolates on asparagine was only about 20% lower than that for the olive isolates, among the subisolates it was about 57% lower. Unfortunately, we did not include glutamine in the second test, as this was the only substrate on which a difference in oxygen uptake occurred on the first test.

Utilization of sucrose.—The foregoing results might suggest that *P. savastanoi* cannot utilize sucrose as a carbon source. Nevertheless, in a medium containing this carbohydrate as a sole carbon source, some isolates grew and produced detectable amounts of acid. For example, when five isolates from olive and six from oleander were inoculated into the basal medium containing sucrose as sole carbon source, only one of the olive isolates (023) grew and produced acid, whereas all but one of the oleander isolates (N16-O) did so (Table 2). It should be noted that N16-O, although originally obtained from oleander, had recently been inoculated into olive and reisolated. In contrast, culture 08-N, originally from olive but recently inoculated into oleander and reisolated, failed in all four tests to grow and produce measurable amounts of acid on sucrose.

Since certain isolates grew and produced acid when cultured in sucrose, it appeared that certain

TABLE 1. Oxidative rates, QO_2N[a], of olive and oleander isolates of *Pseudomonas savastanoi* on various carbohydrates and amino acids

Carbon compound	25 stock isolates[b] from		25 sub-isolates[c] from	
	Olive	Oleander	Olive	Oleander
Glucose	203	126	177	190
Fructose	287	285	345	352
Sucrose	7	12	14	12
Galactose	46	56		
Xylose	7	30		
Glycerol	159	195		
Asparagine	88	70	123	52
Glutamine	180	127		
Alanine	414	364	455	372
Serine	226	213	227	198
Phenylalanine	46	24		
Tryptophan	14	34		

[a] Microliters of O_2 taken up per hr per mg of nitrogen.
[b] Single-cell isolates obtained from different localities in California.
[c] Single-colony subisolates from one olive and one oleander source.

TABLE 2. Acid production[a] from sucrose by isolates of *Pseudomonas savastanoi* from olive and oleander

	Olive isolates					Oleander isolates					
Test[b]	06	08	020	023	08-N	N15	N16	N20	N16-0	N15-N	N21
1	–	–	–	+	–	–	+	+	–	–	–
2	–	–	–	+	–	+	+	+	–	+	+
3	–	–	–	+	–	+	+	+	–	+	+
4	–	–	–	+	–	+	+	+	–	+	+

[a] Initial pH 7.1. Isolates marked "+" reduced pH to 6.1 or below; those marked "–" did not reduce the pH below 6.9.
[b] Nitrogen source was ammonium sulfate.

TABLE 3. Oxygen uptake on sucrose and β-fructofuranosidase activity of oleander and olive isolates after growing in a medium with glucose or sucrose as an energy source

	Grown on sucrose		Grown on glucose	
Isolate	QO_2 (N)	Enzyme activity[b]	QO_2 (N)	Enzyme activity[b]
Olive				
06	[c]		8	<.02
020			5	<.02
025			6	<.02
023	190	1.8	0.23	<.02
Oleander				
N15	240	1.7	2	<.02
N16	71	1.0	7	<.02
N20	240	1.3	11	<.02
N16-0			<0.2	<.02

QO_2 (N) values are calculated from oxygen uptake on sucrose in respirometric experiments with cells grown either on sucrose or glucose.
[b] Enzyme activity is expressed as μmoles of glucose released per minute per mg of protein with sucrose as the substrate in the β-fructofuranosidase assay. Enzyme preparations were from 24-hr cultures in sucrose or glucose medium A.
[c] No growth occurred in these cultures.

systems were induced that permitted utilization of sucrose. For example, when various oleander isolates were first grown in a medium with sucrose as the carbon source and then tested in the respirometer with sucrose, rapid oxygen uptake was observed (Table 3). Those results further suggested that sucrose utilization is associated with production of an inducible enzyme.

Identity of the system that degrades sucrose.—Since all isolates from oleander or olive readily respired glucose and fructose, it appeared that inability to grow on sucrose could be associated with absence of a system that breaks the compound down to one or both of its monosaccharide components. To determine the route of sucrose utilization, sucrose-^{14}C was incubated with cell-free preparations of isolates 023, N15, N16, and N20 that had been grown on sucrose. In each case, analysis for radioactive reaction products by chromatographic strip counting revealed the presence of two radioactive compounds that cochromatographed with authentic glucose and fructose (Fig. 1), and that equal amounts of the two products were formed as sucrose degradation occurred. Only the results with isolate N15 are shown in Fig. 1, but similar results were obtained with the other three isolates. By scintillation counter measurements of the radioactivity associated with each compound shown in Fig. 1, we determined that 64.5 μmoles of sucrose had been utilized during the 15-min incubation period. When we assumed that the two products were glucose and fructose, we calculated that 64.2 μmoles of the former and 66.2 μmoles of the latter were formed during the same period of incubation. The identity of the products was subsequently established by (i) specific enzymatic assays which detected D-fructose (64 μmoles) and D-glucose (68 μmoles) in the 15-min reaction mixture; and (ii) the phenylhydrazine reaction by which the products of the reaction yielded yellow crystals that melted at 204-205 C. The reported melting point for glucosazone is 205 C (20).

We concluded that sucrose was hydrolyzed to yield stoichiometric amounts of glucose and fructose, and the cleavage of sucrose was catalyzed by a β-fructofuranosidase (invertase).

Induction of β-fructofuranosidase.—Glucose-grown olive and oleander isolates that failed to respire sucrose were examined for β-fructofuranosidase activity. The specific activity of β-fructofuranosidase

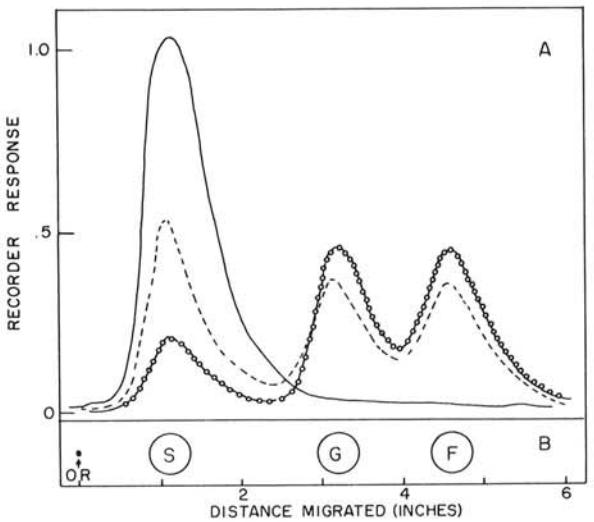

Fig. 1. A) A composite drawing of charts from a recorder attached to a chromatographic strip scanner through which were passed strips of chromatograms bearing radioactive components in a reaction mixture with sucrose-U-^{14}C and enzyme preparation from sucrose-grown *Pseudomonas savastanoi* isolate N15. Components in the mixture with heat-inactivated enzyme preparation (————); components in a reaction with active enzyme preparation after 5 min (– – –), after 15 min (o – o – o – o –). **B)** Distance migrated (inches) of authentic samples of sucrose (S), glucose (G), and fructose (F). OR = the point of application of the samples prior to development in *n*-butanol-glacial acetic acid-water (4:1:5, v/v), organic phase.

in these isolates was less than 0.02 (Table 3). However, five of the eight isolates tested grew on sucrose, and specific activity of the enzyme from these isolates ranged from 0.23 to 0.37. The two isolates, N16-O and 023, gave results that were consistent with their behavior in the sucrose utilization experiments. Transfer of glucose-grown cells to a complete medium with sucrose as the carbon source resulted in an increase in specific activity of the enzyme (Fig. 2). It should be noted that no increase in β-fructofuranosidase activity was detectable in the bacteria for the first 30 min after transfer of the cells to the sucrose medium. Also during this interval of time, no increase in cell number was evident. After 30 min, however, there occurred a steady increase in cell numbers, accompanied by a rapid increase in specific activity of the enzyme. In contrast, the bacteria that were transferred to the glucose medium appeared to resume immediate growth, accompanied by only a slight increase in specific activity of β-fructofuranosidase. The average doubling time of the bacterium in both media was estimated to be 92 min at 25 C.

Specific activity of the enzyme in preparations of a few glucose-grown oleander isolates not described in Table 1, however, were as high as 0.08. Upon transfer of such isolates to sucrose, specific activity of the enzyme increased as much as 3-fold.

Sucrose transport.—Failure of whole cells to respire sucrose could also be associated with lack of uptake of the sugar by the cells. Accordingly, sucrose transport studies were carried out with several isolates under culture conditions identical to those of the preceding experiment. The data shown in Table 4 reveal that all the isolates tested showed low capacity for sucrose uptake when grown on D-glucose. After transfer to a medium containing sucrose, sucrose transport rates were high in the three oleander isolates that were tested, but remained unchanged in the two olive isolates (Table 4). In addition, it appears that glucose represses appearance of the sucrose transport system since reduced transport rates were obtained with bacteria grown in a medium containing both glucose and sucrose.

DISCUSSION.—The bacteria from both olive and oleander seem to be characterized in respirometer experiments by a high uptake of oxygen on alanine, fructose, glucose, and serine, and much lower uptake on sucrose, galactose, xylose, phenylalanine, and tryptophan. The bacteria from the two hosts seem to differ, however, in utilization of sucrose. Judging from the lack of oxygen uptake in respirometer experiments, the bacteria from neither source

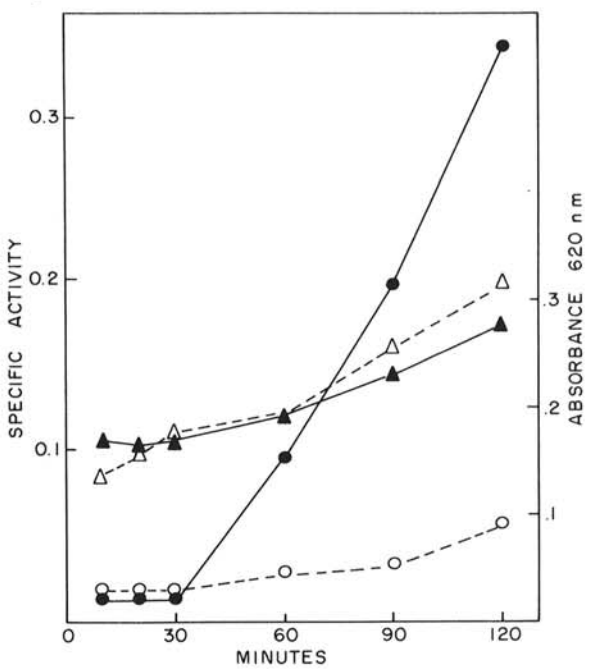

Fig. 2. β-Fructofuranosidase and growth of *P. savastanoi* isolate N15 as a function of time after transfer to sucrose medium A. Specific activity of β-fructofuranosidase after transfer to sucrose (●————●) or glucose medium A (○ – – ○) and increase in cell numbers of the bacterium, as determined by light-scattering measurement at 620 nm, as a function of time (minutes) after transfer. β-Fructofuranosidase assays were carried out at pH 7 which was determined to be the pH optimum for the reaction under conditions described in MATERIALS AND METHODS. An absorbance of 0.100 at 620 nm approximates 10 cells/ml. (– ▲ –), absorbance of sucrose medium A culture; (– △ – –), absorbance of glucose medium A culture.

TABLE 4. Sucrose transport in oleander and olive isolates of *Pseudomonas savastanoi*

Isolate tested	Sucrose transport rates[a]			
	At zero time[b]	After 90 min in medium with[c]		
		Sucrose	Sucrose-glucose	Glucose
Oleander				
N16	0.05	0.29	0.06	0.06
N15	0.06	0.18	0.08	0.05
N20	0.04	0.45	0.25	0.07
Olive				
025	0.02	0.05	0.07	0.05
08	0.07	0.04	0.08	0.04
023	0.02	0.20		0.02

[a] Transport rates are expressed as μmoles of sucrose taken up per gram dry weight of cells per 30 sec at 25 C by procedures described in MATERIALS AND METHODS.

[b] The rate of sucrose transport by cells after 24 hr in glucose medium A prior to transfer to the 3 different media indicated in the 3 columns on the right.

[c] Rates of sucrose transport by whole cells 90 min after transfer of cells from the 24-hr culture in glucose medium A to the three different media indicated.

possessed a constitutive enzyme for sucrose utilization under the conditions of the experiment. Yet, in a complete medium in which sucrose was the only carbon source, most of the oleander isolates grew well, as judged by a significant reduction in pH. Further experiments indicated that, given the right conditions, those isolates were capable of producing the systems necessary to the utilization of sucrose, whereas all but one of the olive isolates (023) apparently were not. Similarly, one oleander isolate (N16-O) that had been inoculated into olive and reisolated lacked the capacity to utilize sucrose. These exceptions bear further investigation.

In the β-fructofuranosidase induction experiments, production of the enzyme is rapid after a lag period of 30 min (Fig. 2). Recent evidence indicates that capacity for sucrose transport precedes appearance of β-fructofuranosidase (T. Kosuge, *unpublished data*). Therefore, it was expected that the same isolate incubated with sucrose in respirometer experiments would show a similar lag period of low O_2 uptake that would be followed by rapid increase in O_2 consumption. However, such respirometer measurements conducted up to 60 min showed no significant O_2 uptakes. It is undoubtedly significant that the medium used for the β-fructofuranosidase experiment contained all ingredients for growth of the organism, whereas that used in the respirometer experiment lacked a nitrogen source and contained only the sucrose and potassium phosphate buffer. Under the latter conditions, the organism appeared not to synthesize the systems necessary for sucrose utilization. D-Glucose may also repress induction of β-fructofuranosidase since specific activities of the enzyme in cells transferred to glucose-sucrose medium were 20 to 48% lower than in cells transferred to sucrose medium A. The results of these investigations suggest that utilization of sucrose by the various isolates depends upon induction of a sucrose transport system for entry of sucrose into the cell and induction of a β-fructofuranosidase that catalyzes the degradation of sucrose to glucose and fructose. The capacity to synthesize both systems appears to be lacking in most olive isolates, but is characteristic of oleander isolates. Whether this characteristic consistently separates the bacteria from the two hosts remains to be determined.

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